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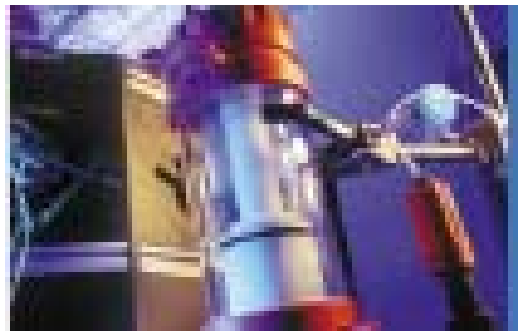
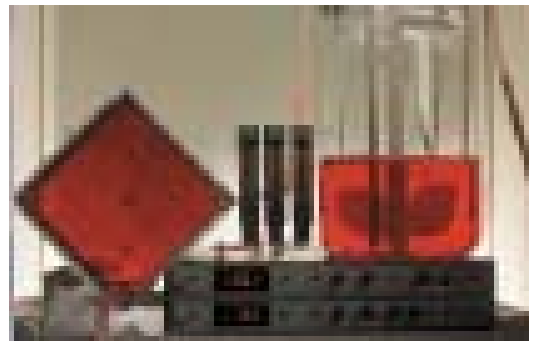




PRACTICAL ASSAY ISSUES FOR PERT

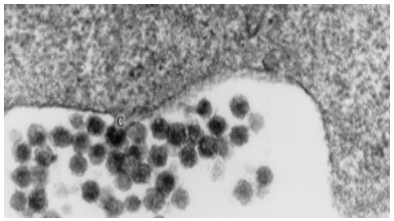
Vaccine Cell Substrate 2004

June 29th, 2004

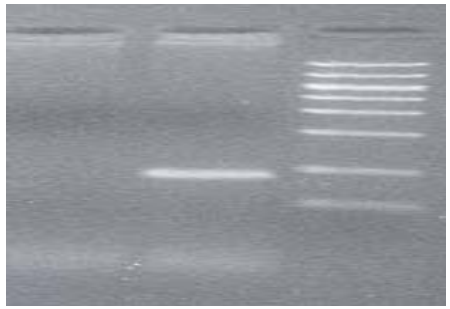
Audrey Chang, Ph.D.

Rockville, MD 20820

Assays to detect the presence of Retrovirus



Electron Microscopy



Viral gene specific PCR

Virus Propagation

Reverse Transcriptase

Retroviruses: frequent viral contaminant in cell substrates (Moore, 1992, Losikoff, 1992)

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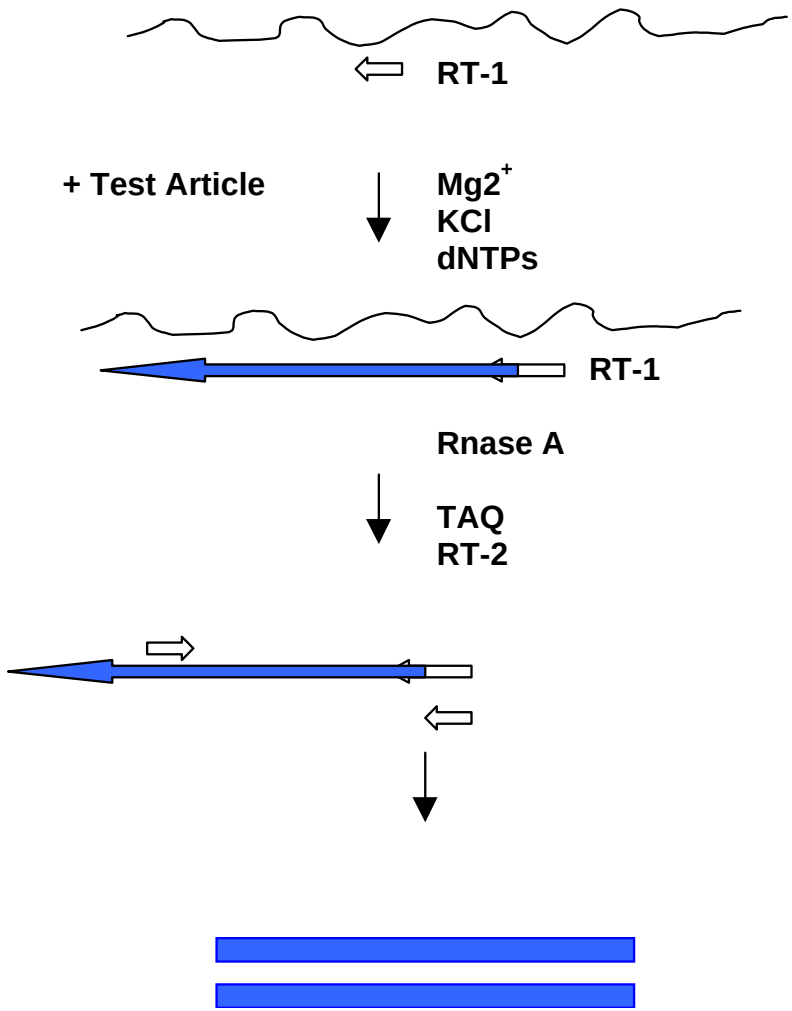
Highly sensitive RT assays

- Silver *et al* (1993)
  - "PBRT"
- Pyra *et al* (1994)
  - "PERT"
- Heneine *et al* (1995)
  - "Amp-RT"



RT assay coupled with a PCR endpoint

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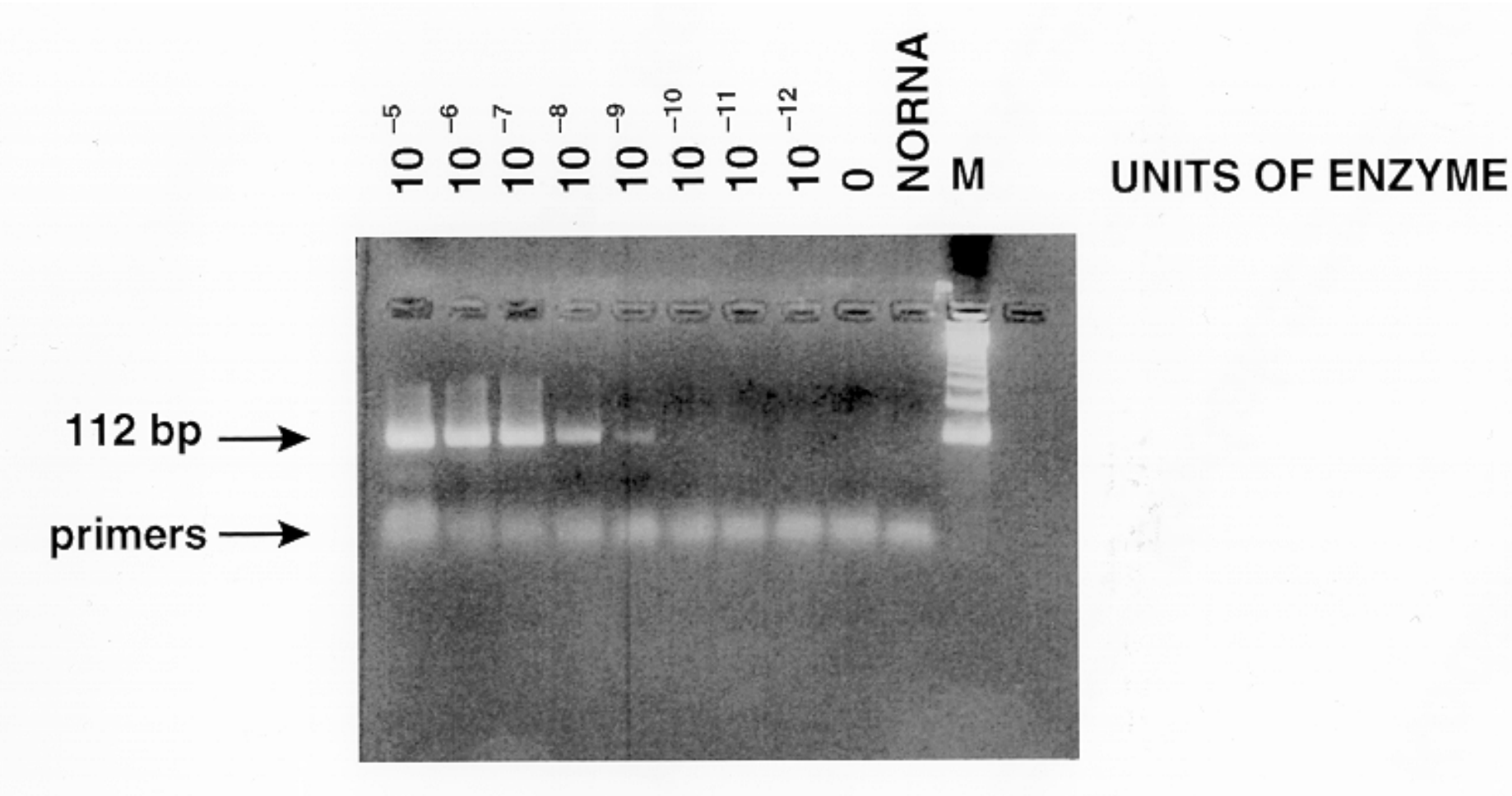
PERT assay titration of RT



10^6 increase in detection sensitivity from conventional RT method

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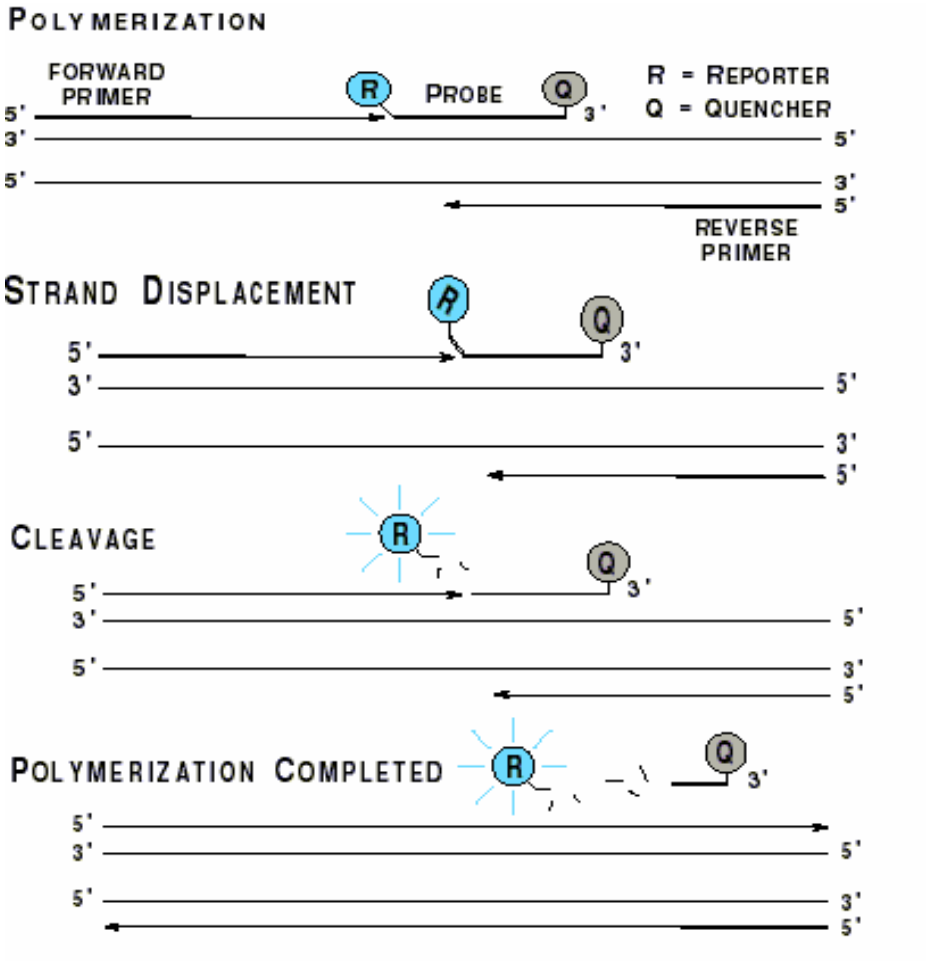
Quantitative - PCR

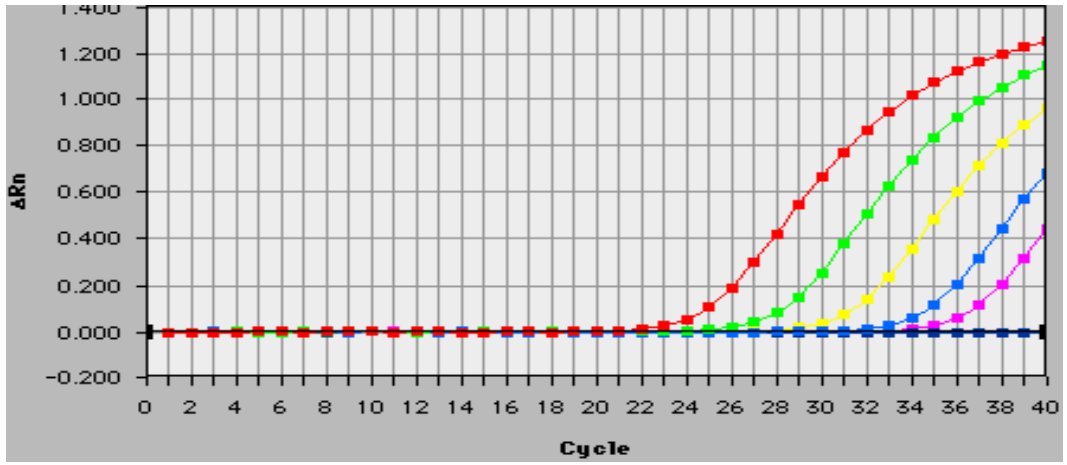
- 5’ nuclease," Real-Time, TaqMan™ PCR assay
- Dual -label probe that generates fluorescence as a function of generated PCR product

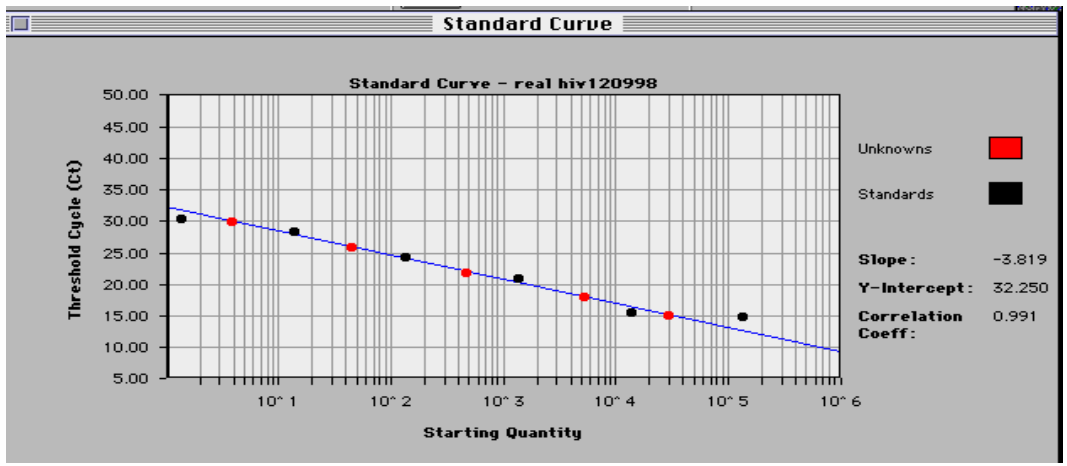


Fluorescent endpoint replaces gel endpoint for PCR readout

Quantitative PERT assay

- Q-PERT
  - **Q**uantitative
- F-PERT
  - **F**luorescent
- TM-PERT
  - **T**aq**M**an
- STF-PERT
  - **S**ingle-**T**ube **F**luorescent
- “PBRT”
  - **P**CR-**B**ased **RT**





Ct = **t**hreshold **c**ycle

Report results in terms of RT units, RT molecules, or RV particles

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F-PERT with HIV particles with commercially available particle # and titer

C_T	RT signal	Virus Particles	$TCID_{50}$
14.0	+	6.0×10^4	5×10^1
15.2	+	6.0×10^3	5×10^0
21.3	+	6.0×10^2	5×10^{-1}
24.5	+	6.0×10^1	5×10^{-2}
28.9	+	6.0×10^0	5×10^{-3}
31.0	+	6.0×10^{-1}	5×10^{-4}
36.5	+	6.0×10^{-2}	5×10^{-5}
39.1	+/-	6.0×10^{-3}	5×10^{-6}
40.00	-	No RNA	n/a
40.00	-	No RT	n/a

Multiple RT molecules per virus particle

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PERT : Broad Detection Range

Virus or RT species	Source	Results
Foamyvirus	Infected cells	+
HTLV I	Infected cells	+
GaLV	Infected cells	+
SIV	Infected cell supernatant	+
SRV 2	Infected cell supernatant	+
SMRV	Purified virus	+
EIAV	Purified virus	+
R-MLV	Purified virus	+
MMLV	Purified virus	+
AMV	Purified RT	+
HIV	Purified RT	+
MMLV	Purified RT	+
Adenovirus	Purified virus	-

PERT will detect RT activity from all Retrovirus families

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Features of PERT (F-PERT)

- 10^6 increase in sensitivity over conventional RT test
- Wide detection range (all retroviruses have RT activity)
  - Unknown (unsequenced) Retroviruses
  - Non-cytopathic retrovirus
  - Non-infectious retrovirus (endogenous RV)
- Broad applications
  - Blood donor screening
  - Medical/veterinary diagnosis
  - Disease monitoring
  - Biomedical research
  - **Product safety testing**

December 18th, 1998

FDA CBER "Letter to Viral Vaccine IND sponsors – Use of PCR-based Reverse Transcriptase Assay"

- *Requests manufacturers of viral vaccines for human used produced in mammalian and avian cells and all other vaccines also containing animal materials to be tested for RT activity by the PERT assay*

- State of the art methodology

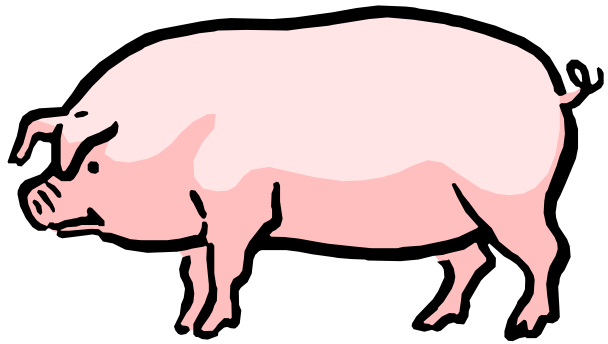
F-PERT assay

- Clarified cell culture supernatants from healthy cultures (log phase), minimal cellular debris
- Lysis by freeze-thaws in the presence of aprotinin, leupeptin, and pepstatin at pH 8
- Assay controls include
  - *Negative controls:* contains all reaction components except (RT or RNA) to verify the absence of (RT or RNA) contamination in assay reagents
  - *Positive control:* contains all reaction components and RT at ten fold dilutions to generate standard curve
  - *Spiked sample:* Contains the test sample with a spike of RT to assess potential matrix inhibition presence in the sample (e.g serum-free media components)

Non-cytopathic retroviruses

Pig Endogenous RetroVirus

- 50 copies/genome
- C-type virus endogenous virus
- Human tropic PERV
  - isolated pig kidney cells (PK-15) capable of growing in human cells (293)
  - non-cytopathic
  - Xenotransplantation concern



PERV: non-cytopathic retrovirus

PK(15) + Mitomycin C

↓

293 cell monolayer (8 passages at

↓

culture supernatant

↓

293 cell monolayer (1 passage)

↓

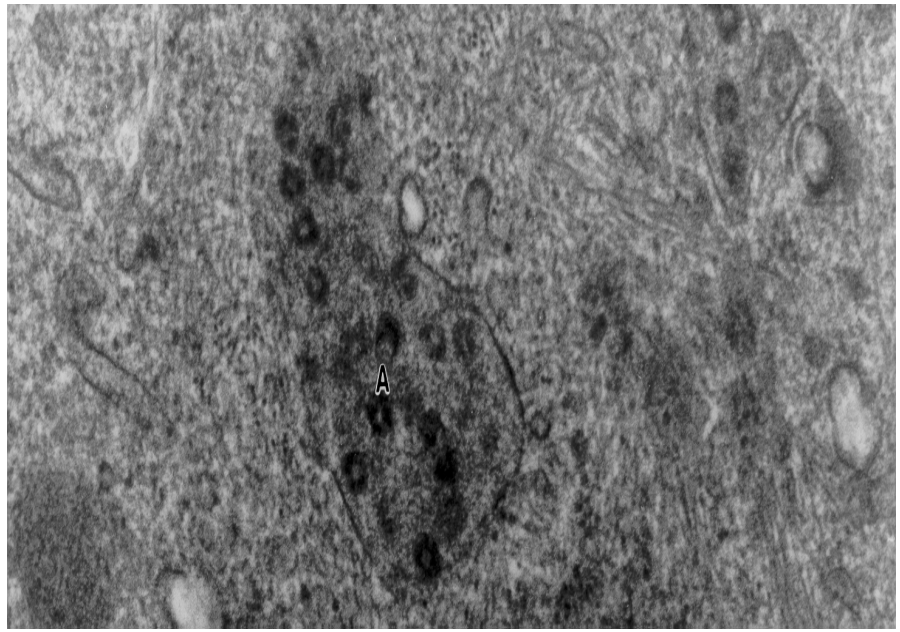
culture supernatant

↓

293 monolayer (7 passages 29 day

↓

cell free PERV stock



PERT virus purification using PERT as the "titer" in RT units

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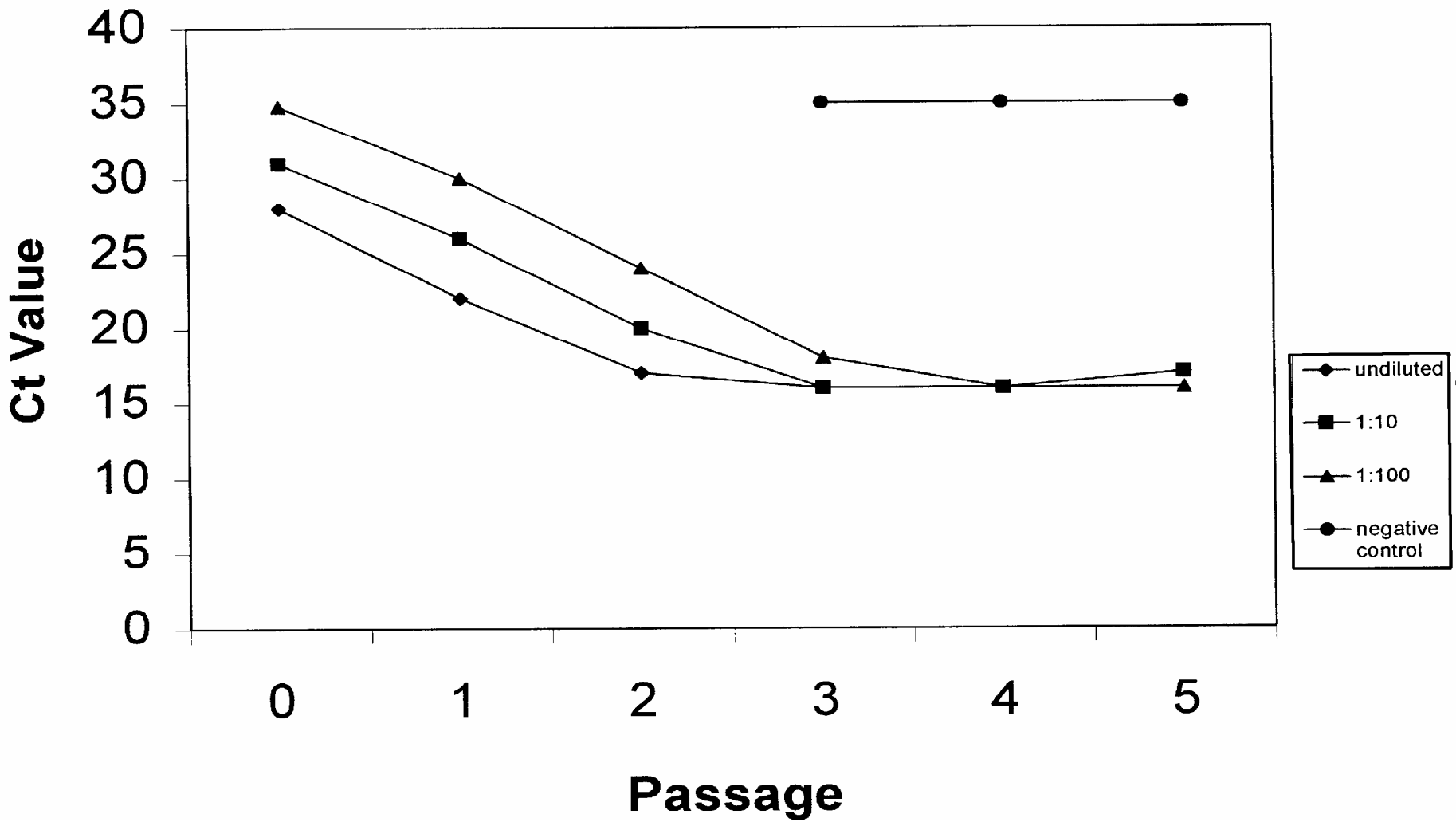
Kinetics of PERV infections in 293 cells as assayed Q-PERT



F-PERT detects presence of infectious PERV

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Two categories of "False Positives"

(1) PERT positive due to contamination

RT, DNA amplicon, RV, DNA polymerase

(2) PERT positive due to RT activity from non-infectious entities that encode authentic RT (e.g. defective retrovirus, endogenous retrovirus, ancient retrovirus, retrovirus-like particles, RVLP, transposons)

Certain rodent, insect, and avian cell lines possess endogenous retroviral particles that express RT activity and will test positive for RT in PERT

PERT assay will detect RT from non-infectious particles

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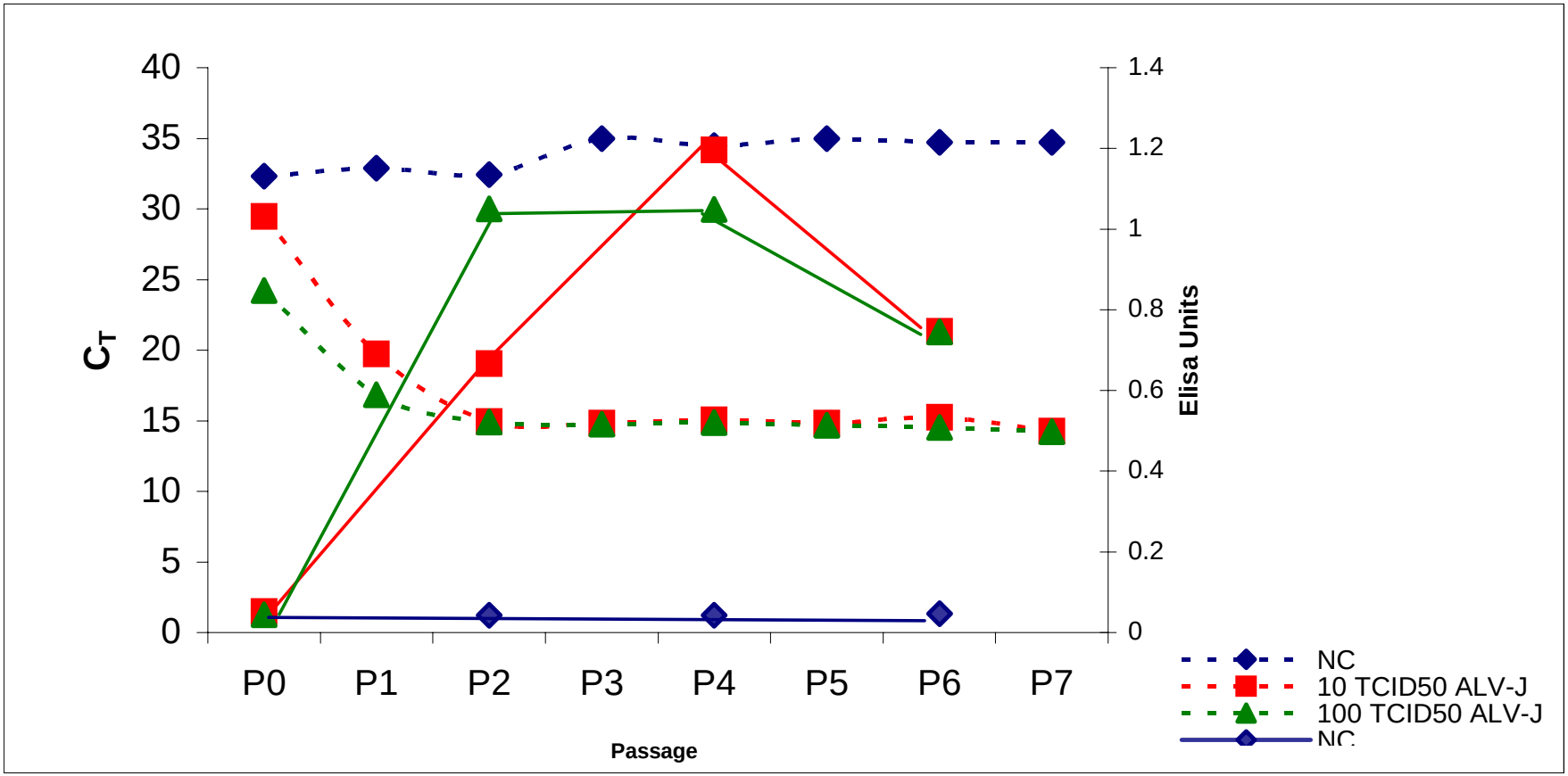
F-PERT for detection of infectious virus ALV-J in TEF cells



Increase in RT levels over endogenous RT levels correlates to presence of infectious virus

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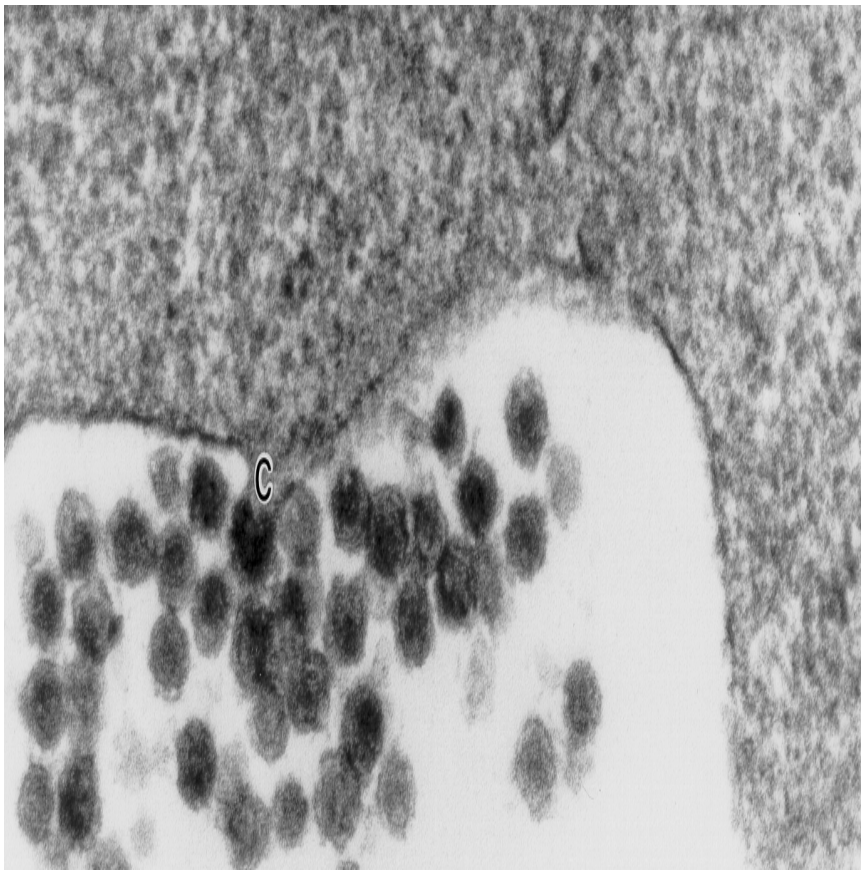
CHO: Endogenous Retrovirus-like Particles



- Type C-like retrovirus
- band 1.13-1.06 g/ml in sucrose gradients
- 100-300 copies/genome
- Multiple stop codons in ORF (endonuclease)
- small percentage considered infectious
- “defective” “RetroVirus-Like-Particle (RVLP)

RVLP resemble infectious RV and are a safety and regulatory concern

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Recent Publications

Biologicals (2000) **28**, 137–148
doi:10.1006/biol.2000.0250, available online at http://www.idealibrary.com on IDEAL®

Real-time Quantitative PCR for Retrovirus-like Particle Quantification in CHO Cell Culture

Christina de Wit[1], Corinne Fautz[2] and Yuan Xu[1]*
[1]*Department of Cell Culture and Fermentation R&D,* [2]*Department of Quality Control, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, U.S.A.*

Biologicals (2001) **29**, 000–000
doi:10.1006/biol.2001.0290, available online at http://www.idealibrary.com on IDEAL®

Evaluation of a Quantitative Product-enhanced Reverse Transcriptase Assay to Monitor Retrovirus in mAb Cell-culture

Kurt Brorson[1]*, Yuan Xu[2], Patrick G. Swann[1], Elizabeth Hamilton[1], Mehnaz Mustafa[1], Christina de Wit[2], Lenore A. Norling[2] and Kathryn E. Stein[1]
[1]*Division of Monoclonal Antibodies, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, MD 20892, USA;* [2]*Genentech, Inc., 1 DNA Way, South San Franciso, CA 94080, USA*

Q-PCR and F -PERT to enumerate retrovirus particles

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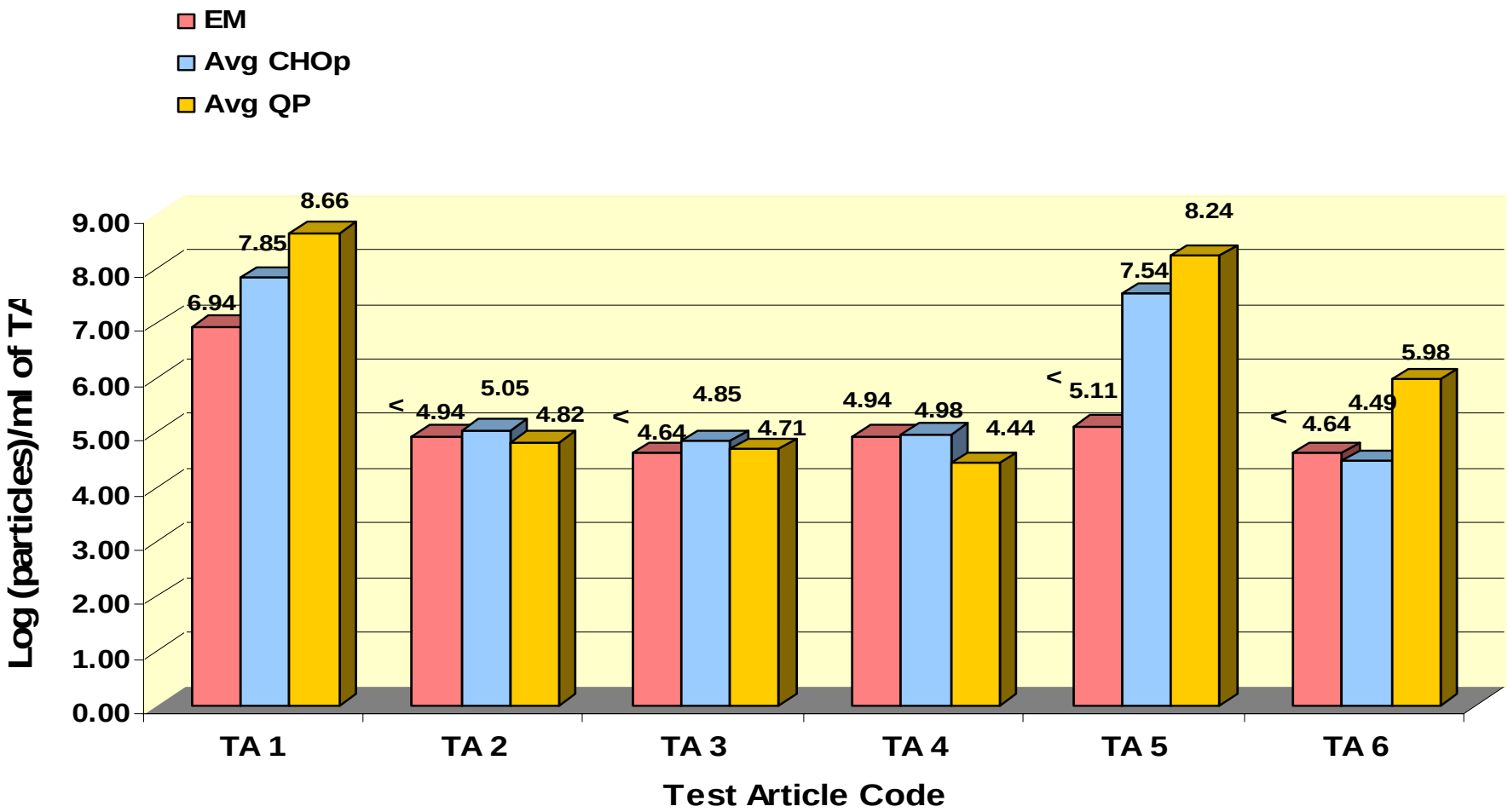
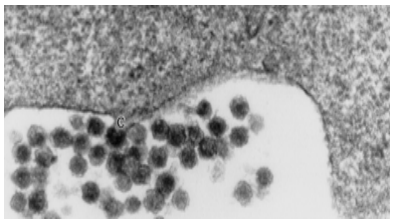
Comparison of EM, PCR, and PERT

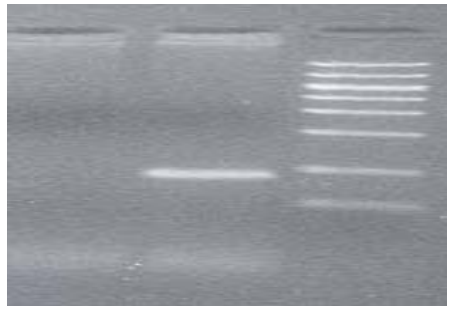


F-PERT assay to replace EM for particle enumeration

Product Safety: Absence of Retrovirus



Electron Microscopy



Viral gene specific PCR

Virus Propagation

Reverse Transcriptase

PERT assays

1. ***Adventitious RV –broad spectrum***
2. ***Characterization of RT activity (infectious vs endogenous)***
3. ***Detecting and Quantitating Viral Particle Load (alternative to EM)***

PERT assay now part of the panel of assays for retrovirus screening